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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: DNA SEQUENCING BY MULTIPLE MIXED OLIGONUCLEOTIDE PROBES

(57) Abstract

A method is provided for sequencing nucleic acids without the need to separate similarly sized DNAs or RNAs by gel electrophoresis. The method relies on the separate hybridization of multiple mixed oligonucleotide probes to a target sequence. The mixed oligonucleotide probes comprise sequences of fixed and non-fixed bases corresponding to every possible permutation of fixed and non-fixed bases less than or equal to the length of the probes. For each probe, the hybridizations provide the number of times the probe's particular sequence of fixed bases appears in the target sequence. The target sequence is then mathematically reconstructed from this data and a knowledge of the probe sequences.

CGK00033902

DNA SEQUENCING BY MULTIPLE MIXED OLIGONUCLEOTIDE PROBES

Background

The ability to determine DNA sequences is crucial for understanding the function and control of genes and for applying many of the basic techniques of molecular biology. Native DNA consists of two linear polymers, or strands of nucleotides. Each strand is a chain of nucleosides linked by phosphodiester bonds. The two strands are held together in an antiparallel orientation by hydrogen bonds between complementary bases of the nucleotides of the two strands: deoxyadenosine (A) pairs with thymidine (T) and deoxyguanosine (G) pairs with deoxycytidine (C).

Presently there are two basic approaches to DNA sequence determination: the dideoxy chain termination method, e.g. Sanger et al, Proc. Natl. Acad. Sci., Vol. 74, pgs. 5463-5467 (1977); and the chemical degradation method, e.g. Maxam et al, Proc. Natl. Acad. Sci., Vol. 74, pgs.560-564 (1977). The chain termination method has been improved in several ways, and serves as the basis for all currently available automated DNA sequencing machines, e.g. Sanger et al, J. Mol. Biol., Vol. 143, pgs. 161-178 (1980); Smith et al, Nucleic Acids Research, Vol. 13, pgs. 2399-2412 (1985); Smith et al, Nature, Vol. 321, pgs. 674-679 (1987); Prober et al, Science, Vol. 238, pgs. 336-341 (1987), Section II, Meth. Enzymol., Vol. 155, pgs. 51-334 (1987),and Church et al, Science, Vol 240, pgs. 185-188 (1988).

Both the chain termination and chemical degradation methods require the generation of one or more sets of labeled DNA fragments, each having a common origin and each terminating with a known base. The set or sets of fragments must then be separated by size to obtain sequence information. In both methods, the DNA fragments are separated by high resolution gel electrophoresis. Unfortunately, this step severely limits the size of the DNA chain that can be sequenced at one time. Non-automated sequencing can

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accommodate a DNA chain of up to about 500 bases under optimal
conditions, and automated sequencing can accommodate a chain
of up to about 300 bases under optimal conditions, Bankier et
al, Meth. Enzymol., Vol. 155, pgs. 51-93 (1987); Roberts,
Science, Vol. 238, pgs. 271-273 (1987); and Smith et al,
Biotechnology, Vol. 5, pgs. 933-939 (1987).

This limitation represents a major bottleneck for many
important medical, scientific, and industrial projects aimed
at unraveling the molecular structure of large regions of
plant or animal genomes, such as the project to sequence all
or major portions of the human genome, Smith et al,
Biotechnology (cited above).

In addition to DNA sequencing, nucleic acid
hybridization has also been a crucial element of many
techniques in molecular biology, e.g. Hames et al, eds.,
Nucleic Acid Hybridization: A Practical Approach (IRL Press,
Washington, D.C., 1985). In particular, hybridization
techniques have been used to select rare cDNA or genomic
clones from large libraries by way of mixed oligonucleotide
probes, e.g. Wallace et al, Nucleic Acids Research, Vol. 6,
pgs. 3543-3557 (1979), or by way of interspecies probes, e.g.
Gray et al, Proc. Natl. Acad. Sci., Vol. 80, pgs. 5842-5846
(1983). Nucleic acid hybridization has also been used to
determine the degree of homology between sequences, e.g.
Kafatos et al, Nucleic Acids Research, Vol. 7, pgs. 1541-1552
(1979), and to detect consensus sequences, e.g. Oliphant et
al, Meth. Enzymol., Vol. 155, pgs. 568-582 (1987). Implicit
to all of these applications is the notion that the known
probe sequences contain information about the unknown target
sequences. This notion apparently has never been exploited to
obtain detailed sequence information about a target nucleic
acid. In view of the limitations of current DNA sequencing
methods, it would be advantageous for the scientific and
industrial communities to have available an alternative
method for sequencing DNA which (1) did not require gel
electrophoretic separation of similarly sized DNA fragments,
(2) had the capability of providing the sequence of very long

DNA chains in a single operation, and (3) was amenable to
automation.

Disclosure of the Invention

The invention is directed to a method for determining
the nucleotide sequence of a DNA or an RNA molecule using
multiple mixed oligonucleotide probes. Sequence information
is obtained by carrying out a series of hybridizations whose
results provide for each probe the number of times the
complement of the probe's sequence occurs in the RNA or DNA
whose sequence is to be determined. The nucleotide sequence
of the RNA or DNA is reconstructed from this information and
from a knowledge of the probes' sequences. The nucleic acid
whose sequence is to be determined is referred to herein as
the target sequence.

The mixed oligonucleotide probes of the invention are
selected from a set whose members' sequences include every
possible complementary sequence to subsequences of a
predetermined length within the target sequence. The series
of hybridizations are separately carried out such that one or
more of the probes selected from the set are combined with
known quantities of the target sequence, e.g. on a
nitrocellulose filter, or like substrate, under conditions
which substantially allow only perfectly matched probe
sequences to hybridize with the target sequence. Probe
sequences having mismatched bases are substantially removed,
e.g. by washing, and the quantity of perfectly matched probe
remaining hybridized to the target sequence is determined.

In one embodiment of the invention, the set of probes
comprises four subsets. Each of the four subsets contains
probes representing every possible sequence, with respect to
the size of the probe (which is predetermined), of only one of
the four bases. For example, the first subset can contain
probes where every possible sequence of G is represented; the
second subset can contain probes where every possible sequence
of T is represented; and so on for C and A. If the probes

were each 8 bases long, a member probe of the adenosine subset
can be represented as follows:

$$3'-AA \begin{pmatrix} C \\ G \\ T \end{pmatrix} \begin{pmatrix} C \\ G \\ T \end{pmatrix} A \begin{pmatrix} C \\ G \\ T \end{pmatrix} \begin{pmatrix} C \\ G \\ T \end{pmatrix} A-5'$$
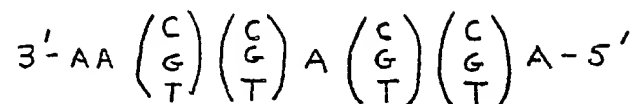
Formula I

The symbol $\begin{pmatrix} C \\ G \\ T \end{pmatrix}$ means that any of the bases C, G, or T

may occupy the position where the symbol is located. Thus,
the above probe has a multiplicity, or degeneracy, of
1x1x3x3x1x3x3x1, or 81. When it is clear from the context
which subset is being considered, the above notation will be
simplified to AA00A00A, where A represents deoxyadenosine and
0 represents the absence of deoxyadenosine.
    Preferably, base analogs are employed in the
oligonucleotide probes whose base pairing characteristics
permit one to reduce the multiplicity of the probe. For
example, in the probe of Formula II, because deoxyinosine (I)
forms nearly equally strong base pairs with A and C, but
forms only a weak or destabilizing base pair with either G or
T, deoxyinosine can replace G and T in the probe, Martin et
al, Nucleic Acids Research, Vol. 13, pgs. 8927-8938 (1985).
Thus, a probe equivalent to that of Formula I, but which has
a much lower multiplicity (i.e. only 16) can be represented as
follows:

$$3'-AA \begin{pmatrix} I \\ C \end{pmatrix} \begin{pmatrix} I \\ C \end{pmatrix} A \begin{pmatrix} I \\ C \end{pmatrix} \begin{pmatrix} I \\ C \end{pmatrix} A-5',$$
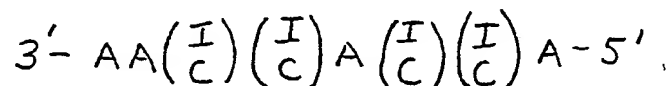
Formula II

Generally, base analogs are preferred which form strong base
pairs (i.e., comparable in binding energy to the natural base
pairs) with two or three of the four natural bases, and a weak
or destabilizing base pair with the complement of a fixed
base (defined below). Such base analogs are referred to

herein as degeneracy-reducing analogs.

It is not critical that the probes all have the same
length, although it is important that they have known
lengths and that their sequences be predetermined. Generally,
the probes will be fixed at a predetermined number of
positions with known bases (not necessarily of the same kind),
e.g. as the A in Formula I, and the remaining positions will
each be filled by a base randomly selected from a
predetermined set, e.g. T, G, and C as in Formula I, or I and
C as in Formula II. The positions in a probe which are non-
degenerate in their base pairing, i.e. have only a single
natural base, are referred to herein as fixed positions. The
bases occupying fixed positions are referred to herein as
fixed bases. For example, the fixed bases in the probes of
Formulas I and II are deoxyadenosine at positions one, two,
five, and eight with respect to the 3' end of the probe.

Generally, sets and/or subsets of the invention each
contain at least one probe having a sequence of fixed and non-
fixed positions equivalent to that of each permutation of a
plurality of fixed and non-fixed positions less than or equal
to the length of the probe. That is, an important feature of
the invention is that the probes collectively contain
.subsequences (up to the total length of the probe) which
correspond to every possible permutation of fixed and non-
fixed positions of each of a plurality of combinations of
fixed and non-fixed positions, the plurality including
combinations containing from zero to all fixed positions.
For example, consider a subset of probes of the invention that
consists of 8-mer probes whose fixed positions contain only
deoxyadenosine and whose initial (i.e., 3'-most) position is
fixed. The probes of Formulas I and II are members of such
a subset. Within such a subset, there is a least one probe
having a subsequence of fixed and non-fixed positions in
positions 2 through 8 which corresponds to each possible
permutation of fixed and non-fixed positions for subsequences
having no fixed positions (one such permutation: A0000000),
one fixed position (seven such permutations, e.g. A000A000),

two fixed positions (twenty-one such permutations, e.g.
A00AA000), three fixed positions (thirty-five such
permutations, e.g. A0000AAA), four fixed positions (thirty-
five such permutations, e.g. A0AAAA00), five fixed positions
(twenty-one such permutations, e.g. AAA00AAA), six fixed
positions (seven such permutations, e.g. AAAA0AAA), and seven
fixed positions (one such permutation: AAAAAAAA). Thus,
the subset has at least 1 + 7 + 21 + 35 + 35 + 21 + 7 + 1 =
128 members.

     The presence of one or more predetermined known sequence
regions in the target sequence   facilitates the
reconstruction of the target sequence. Accordingly, in a
preferred embodiment, the target sequence contains one or more
regions of known sequence, these regions being referred to
herein as known sequence regions. More preferably, the target
sequence contains a first and a second known sequence region,
the first and second known sequence regions being positioned
on opposite ends of the region of the target sequence
containing the unknown sequence of nucleotides. This unknown
sequence of nucleotides is referred to herein as the unknown
sequence region. Most preferably, the first and second known
sequence regions are at least the length of the longest probe
sequence.

I. Composition and Labeling of the Probes

   Mixed oligonucleotide probes for the invention are preferably
synthesized using an automated DNA synthesizer, e.g. Applied
Biosystems (Foster City, CA) models 381A or 380B, or like
instrument. At non-fixed positions mixtures of the appropriate
nucleotide precursors are reacted with the growing
oligonucleotide chain so that oligonucleotides having different
different bases at that position are synthesized
simultaneously, e.g. as disclosed by Wallace et al, Nucleic
Acids Research, Vol. 6, pgs.3543-3557 (1979), and Oliphant et
al, Meth. Enzymol., Vol. 155, pgs. 568-582 (1987).
The probes may be synthesized by way of any of the available
chemistries, e.g. phosphite triester, Beaucage et al,

Tetrahedron Letters, Vol. 22, pgs. 1859-1862 (1981); Caruthers
et al, U.S. patents 4,415,723, 4,458,066, and 4,500,707;
phosphotriester, Itakura, U.S. patent 4,401,796; hydrogen
phosphonate, e.g. Froehler et al, Nucleic Acids Research, Vol.
14, pgs. 5399-5407 (1986); or the like. Once synthesized,
the oligonucleotides are purified for labeling by well known
techniques, usually HPLC or gel electrophoresis, e.g. Applied
Biosystems DNA Synthesizer Users Bulletin, Issue No. 13-Revised
(April 1, 1987).

Selecting the lengths of the probes is an important aspect
of the invention. Several factors influence the choice of
length for a given application, including (1) the ease with
which hybridization conditions can be manipulated for
preferentially hybridizing probes perfectly matched to the
target sequence, (2) the ability to distinguish between roughly
integral amounts of perfectly matched probe hybidized to the
target sequence (e.g. if the probe is relatively long so that
the expected frequency of probe sequences perfectly
complementary to the target is low, one may be required to
distinguish (for example) between amounts of probe in the range
of 10, 20, or 30 picomoles--to infer that 1, 2, or 3 copies of
the probe are present on the target; if the probe is
relatively short so that the expected frequency of probe
sequences perfectly complementary to the target is high, one
may be required to distinguish (for example) between amounts of
probe in the range of 110, 120, or 130 picomoles--to infer that
11, 12, or 13 copies of the probe are present on the target;
since the fractional differences between the latter quantities
are small, there may be less confidence in the inferred copy
number); (3) whether probe multiplicity permits hybridization
with reasonable Cot values (longer probes are more degenerate
than shorter probes, and require higher Cot values for
hybridization, the converse is true of shorter probes); (4) the
practicality of carrying out separate hybridizations for each
type of probe (longer probes give rise to larger sets of
probes, as described above); and (5) the tractability of the
sequence reconstruction problem (the greater the number of

copies of each probe type on the target sequence--which is the tendency if shorter probes are employed, the more difficult the reconstruction problem). Probe sizes in the range of 7 to 11 bases are preferred. More preferably, probes sizes are in the range of 8 to 10 bases, and most preferably, probe sizes are in the range of 8 to 9 bases.

Preferably, degeneracy-reducing analogs are employed at the non-fixed positions of the probes to reduce probe multiplicity, or degeneracy. Many synthetic and natural nucleoside and nucleotide analogs are available for this purpose, e.g. Scheit, Nucleotide Analogs (John Wiley & Sons, New York, 1980). For example, degeneracy-reducing analogs include deoxyinosine for use in cytosine or adenosine probes to replace G and T at non-fixed positions, 2-aminopurine for use in cytosine or guanosine probes to replace A and T at non-fixed positions, and N^4- methoxydeoxycytidine, N^4-aminodeoxycytidine, or 5-fluorodeoxyuridine for use in adenosine or guanosine probes to replace T and C. Use of deoxyinosine in oligonucleotide probes is disclosed by Martin et al (cited above); Seela et al, Nucleic Acids Research, Vol. 14, pgs. 1825-1844 (1986); Kawase et al, Nucleic Acids Research, Vol. 14, pgs. 7727-7737 (1986); Ohtsuka et al, J. Biol. Chem., Vol. 260, pgs. 2605-2608 (1985); and Takahashi et al, Proc. Natl. Acad. Sci., Vol. 82. pgs. 1931-1935 (1985). Deoxyinosine phosphoramidite precursors for automated DNA synthesis are available commercially, e.g. Applied Biosystems (Foster City, CA). The synthesis of N^4-methoxycytidine and its incorporation into oligonucleotide probes is disclosed by Anand et al, Nucleic Acids Research, Vol. 15, pgs. 8167-8176 (1987). The synthesis of 2-aminopurine and its incorporation into oligonucleotide probes is disclosed by Eritja et al, Nucleic Acids Research, Vol. 14, pgs. 5869-5884 (1986). The synthesis of 5-fluorodeoxyuridine and its incorporation into oligonucleotide probes is disclosed by Habener et al, Proc. Natl. Acad. Sci., Vol. 85, pgs. 1735-1739 (1988). And the preparation of N^4-aminodeoxycytidine is disclosed by

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Negishi et al, <u>Nucleic</u> <u>Acids</u> <u>Research</u>, Vol. 11, pgs. 5223-
5233 (1983).

Nucleoside analogs are also employed in the invention to
reduce the differences in binding energies between the
various complementary bases. In particular, 2-aminoadenine
can replace thymine in either the probe or target sequences to
reduce the binding energy differences between A-T nucleoside
pairs and G-C nucleoside pairs, e.g. Kirnos et al (cited
above), Chollet et al (cited above), and Cheong et al, <u>Nucleic</u>
<u>Acids</u> <u>Research</u>, Vol. 16, pgs. 5115-5122 (1988). Procedures
for synthesizing oligonucleotides containing 2-aminoadenine
are disclosed by Chollet et al (cited above); Gaffney et al,
<u>Tetrahedron</u>, Vol. 40, pgs. 3-13 (1984), and Chollet et al
<u>Chemica</u> <u>Scripta</u>, Vol. 26, pgs. 37-40 (1986). Likewise, 2-
amino-2'-deoxyadenosine can replace deoxyadenosine to increase
the binding energy at positions where A-T pairs occur, e.g.
Huynh-Dihn et al, <u>Proc.</u> <u>Natl.</u> <u>Acad.</u> <u>Sci.</u>, Vol. 82, pgs. 7510-
7514 (1985).

In some embodiments, it may be preferable to replace a
more degenerate probe with several less degenerate probes
which collectively are capable of obtaining the same
information about the target sequence. For example, consider
the 9-mer probe A00000000. This probe can be replaced by the
three less degenerate probes A0000000C, A0000000G, and
A0000000T. Thus, at the cost of two additional
hybridizations, the degeneracy of the most degenerate probe in
the set is reduced from 256 to 128 (assuming the use of
deoxyinosine at non-fixed positions).

The oligonucleotides of the invention can be labeled in a
variety of ways to form probes, including the direct or
indirect attachment of radioactive moieties, fluorescent
moieties, electron dense moieties, and the like. It is only
important that each sequence within a probe be capable of
generating a signal of the same magnitude, so that
quantitative measurements of probe number can be made.
There are several means available for derivatizing
oligonucleotides with reactive functionalities which, permit
the addition of a label, e.g. Connolly, <u>Nulceic</u> <u>Acids</u>

Research, Vol 15, pgs. 3131-3139 (1987); Gibson et al, Nucleic Acids Research, Vol. 15, pgs. 6455-6467 (1987); Spoat et al, Nucleic Acids Research, Vol. 15, pgs. 4837-4848 (1987); and Mathews et al, Anal. Biochem., Vol. 169, pgs. 1-25 (1988).

In one preferred embodiment, the oligonucleotides of the invention are radioactively labeled with ^{32}P using standard protocols, e.g. Maxim and Gilbert, Meth. Enzymol., Vol. 65, pgs. 499-560 (1980). ^{32}P-labeled probes of the invention are preferably applied to targent DNAs anchored to nitrocellulose, nylon, or the like, at a concentration in the range of about 1-10 ng/ml, and more preferably, in the range of about 1-5 ng/ml. The specific activities of the probe are preferably in the range of about 1-5 X 10^6 cpm/ml.

II. Hybridization

The hybridizations of the probes to the target sequence are carried out in a manner which allows mismatched probe sequences and nonspecifically bound probe sequences to be separated from the duplexes formed between the perfectly matched probe sequences and the target sequence. Usually the separation is carried out by a washing step. Preferably, the first step in the hybridizations is to anchor the target sequence so that washes and other treatments can take place with minimal loss of the target sequences. The method selected for anchoring the target sequence depends on several factors, including the length of the target, the method used to prepare copies of the target, and the like. Preferably, the target sequence is anchored by attaching it to a substrate or solid phase support, such as nitrocellulose, nylon-66, or the like, or such as derivatized microspheres, e.g. Kremsky et al, Nucleic Acids Research, Vol. 15, pgs.2891-2909 (1987).

A known quantity of single or double stranded copies of the target sequence is anchored to the substrate, or solid phase support. As used herein "known quantity" means amounts from which integral numbers of perfectly matched probes can be determined. In some embodiments this means known gram or molar quantities of the target sequence. In

other embodiments, it can mean equal amounts of target
sequence on the plurality of solid phase supports, so that
signals corresponding to integral numbers of probes can be
discerned by comparing signals from the plurality of supports,
or by comparing signals to specially provided standards.
Preferably, the anchoring means is loaded to capacity with the
target sequence so that maximal signals are produced after
hybridization. The target sequence can be prepared in double
stranded form, denatured, and then applied to the anchoring
means, which is preferably a solid phase support, such as
nitrocellulose, GeneScreen, or the like. When the target
sequence is prepared in double stranded form, it is preferably
excised from its cloning vector with one or more
endonucleases which leave blunt ended fragments, e.g. Eco
RV, Alu I, Bal I, Dra I, Nae I, Sma I, or the like. In this
case, both the coding, or sense, strand and the noncoding, or
antisense, strand are sequenced simultaneously. Because of
sequence complementarity, the reconstruction problem is no
more difficult than in the single stranded case.

     Suitable vectors for preparing double stranded target
sequences are those of the pUC series, e.g. Yanisch-Perron et
al, Gene, Vol. 33, pgs. 103-119 (1985). These vectors are
readily modified by adding unique restriction sites to their
polylinker regions. The new unique restriction sites are
selected from restriction endonucleases that leave flush-ended
fragments after digestion. For example, chemically
synthesized fragments containing such sites can be inserted
into the Hind III and Eco RI sites of pUC18 or pUC19. For
these vectors such sites include Bal I, Eco RV, Hpa I, Nae I,
Nru I, Stu I, Sna BI, and Xca I. With the modified pUC, the
precursor of the target sequence (i.e. the unknown sequence
region) can be inserted into a preexisting polylinker site,
e.g. Bam HI; the vector can be amplified and isolated; and the
target sequence can be excised via the restriction
endonucleases that leave flush-ended fragments. The fragments
of the polylinker region excised along with the unknown
sequence region then become the known sequence regions of the

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target sequence.

The preferred method of anchoring DNA to nitrocellulose filters is essentially that described by Kafatos et al (cited above). Up to about 1 ug of target sequence is applied per square millimeter of the filter. Before application the DNA is denatured, preferably in 0.3 to 0.4 N NaOH for about 10 minutes, after which it is chilled with an equal volume of cold water, or optionally cold 2 M ammonium acetate, to a concentration of about 16 ug/ml. Known quantities of the denatured target are spotted onto the filter by carefully controlling the volume of liquid deposited. After each sample is spotted (approximately 1.5 minutes), the filter can optionally be rinsed through with a drop of 1 M ammonium acetate containing about .02-0.2 N NaOH, pH 7.8-9.0. Filters may also be washed with 4xSSC (defined below), e.g. about 200 ml. The filters are air dried, shaken in 2x Denhardt's solution (defined below) for at least 1 hour, drained and air dried again, and baked under vacuum at 80° C for about 2 hours.

Hybridization of the probes to the target sequence usually comprises three steps: a prehybridization treatment, application of the probe, and washing. The purpose of the prehybridization treatment is to reduce nonspecific binding of the probe to the anchoring means and non-target nucleic acids. This is usually accomplished by blocking potential nonspecific binding sites with blocking agents such as proteins, e.g. serum albumin (a major ingredient of Denhardt's solution). For target sequences anchored to nitrocellulose or nylon-66 (e.g. GeneScreen, Nytran, or the like), prehybridization treatment can comprise treatment with 5-10x Denhardt's solution, with 2-6x SSC preferably containing a mild detergent, e.g. 0.5% sodium dodecylsulfate (SDS), for 15 min. to 1 hr. at a temperature in the range of about 25° to 60°. Denhardt's solution, disclosed in <u>Biochem. Biophys. Res. Commun.</u>, Vol. 23, pgs. 641-645 (1966), consists at 10x concentration of 0.2% bovine serum albumin, 0.2% polyvinylpyrolidone, and 0.2% Ficoll. SSC, another standard

reagent in the hybridization art, consists at 1x of 0.15 M
NaCl, 0.015 M sodium citrate, at pH 7.0. Preferred treatment
times, temperatures, and formulations may vary with the
particular embodiment.

Preferably, the probe is applied to the anchored DNA at a
concentration in the range of about 1-10 ng/ml in a solution
substantially the same as the prehybridization solution, e.g.
5-10x Denhardt's solution with 2-6x SSC and a mild
detergent, e.g. 0.5% SDS. More preferably, the probe
concentration is in the range of about 1-5 ng/ml. Preferably,
the hybridization is carried out at a temperature 10-20° C
below the expected 50% dissociation temperatue, T_d, between
the probe and the target. That is, a temperature is selected
at which a high proportion, e.g. greater than 80-90%, of all
the perfectly matched probes form stable duplexes. For 8-mer
probes the preferred hybridization temperature is in the range
of about 10-18° C. Hybridization times are preferably in
the range of about 3-16 hours. Different hybridization times
may be selected for probes of different degrees of degeneracy,
because the effective consentrations of particular sequences
within a highly degenerate probe, e.g. 0A000000, are
considerably less than those of particular sequences in a low
degeneracy probe, e.g. AAA0AAAA. Thus, higher Cot values
(which usually means longer hybridization times) may be
required for more degenerate probes to attain a sufficient
degree of binding of perfectly matched sequences. Preferably,
a single hybridization time is selected for all probes which
is determined by the hybridization kinetics of the most
degenerate probe. Probe degeneracy becomes important when
relative signals are compared after autoradiography. Probes
having higher degeneracy will produce lower signals than
probes of lower degeneracy. Therefore, relative signals
should only be compared among autoradiographs associated with
probes of equivalent degeneracy. Signal comparisons are
aided by the simultaneous running of positive and negative
controls for each probe, or at least for each degeneracy
class.

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Removing nonspecifically bound and mismatched   probe
sequences by washing is an important aspect of the invention.
Temperatures and wash times are selected which permit the
removal of a maximum amount of nonspecifically bound and
mismatched probe sequences, while at the same time permit the
retention of a maximum number of probe sequences forming
perfectly matched duplexes with the target. The length and
base composition of the probe are two important factors in
determining the appropriate wash temperature. For probe
lengths in the preferred range of 7-11 bases, the difference
in duplex stability between perfectly matched and mismatched
probe sequences is quite large, the respective T_d's differing
by perhaps as much as 10° C, or more. Thus, it is not
difficult to preferentially remove mismatches by adjusting
wash temperature. On the other hand, composition differences,
e.g. G-C content versus A-T content, give rise to a broadened
range of probe T_d's, and lower wash temperatures reduce the
ability to remove nonspecifically bound probe. Consequently,
the wash temperature must be maximized for removing
nonspecifically bound probe, yet it cannot be so high as to
preferentially remove perfectly matched probes with relatively
high A-T content.

Preferably, hybridization conditions and/or nucleotide
analogs are selected which minimize the difference in binding
energies of the various base pairs, in order to minimize
sequence-specific differences in probe binding. Such
minimization is preferable because it increases the
sensitivity with which perfectly matched probes can be
detected. Sensitivity is increased because such minimization
makes the transition from probe/target duplexes to single
stranded probe and single stranded target much sharper when
temperature is increased, i.e. the probe/target T_m is less
broad. For example, when hybridization occurs in the presence
of tetraalkylammonium salts, the differences in binding energy
between G-C pairs and A-T pairs is reduced, e.g. Wood et al,
Proc. Natl. Acad. Sci., Vol. 82, pgs. 1585-1588 (1985).
Likewise, use of alpha-anomeric nucleoside analogs results in

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stronger binding energies, e.g. Moran et al, <u>Nucleic Acids</u>
<u>Research</u>, Vol. 16, pgs. 833-847 (1988); and the use of 2-
aminoadenine in place of adenine results in stronger binding,
e.g. Chollet et al, <u>Nucleic Acid Research</u>, Vol 16, pgs. 305-
317 (1988). Accordingly, a preferred wash procedure for 8-mer
probes comprises washing the filters three times for about 15
minutes in 6x SSC containing 0.5% SDS at a temperature in the
range of about 10-12° C, followed by one or two rinses with
3.0 M Me$_4$NCl, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, at a
temperature in the range of about 10-12° C, followed by a 1.5-
2.5 minute wash in 3.0 M Me$_4$NCl, 50 mM Tris-HCl, pH 8.0, 0.5%
SDS, at a temperature in the range of about 24-28° C. For 9-
mer probes, the procedure is substantially the same, except
that the final wash temperature is preferably in the range
of about 26-30°C. After hybridization and washing,
quantitative measurements of bound probe are carried out using
standard techniques, e.g. for radiolabelled probes,
autoradiography or scintillation counting can be used.

III. <u>Sequence Reconstruction</u>

The general nature of the reconstruction problem is
illustrated by the example of Figure 1, in which four subsets
of 4-mer probes are used to analyze the sequence of the 21-
mer, CGAATGGAACTACCGTAACCT. On the left of Figure 1 is a list
of 4-mer probes having every possible permutation of fixed and
non-fixed positions with respect to deoxyadenosine,
deoxycytosine, deoxyguanosine, and thymidine, respectively,
for the following combinations of fixed bases and non-fixed
bases: 1 fixed and 3 non-fixed, 2 fixed and 2 non-fixed, 3
fixed and 1 non-fixed, and 4   fixed and 0 non-fixed. That
is, the list contains at least one probe having a sequence of
fixed and non-fixed positions with respect to A, C, G, and T
equivalent to every possible permutation of A's and non-fixed
positions, C's and non-fixed positions, G's and non-fixed
positions, and T's and non-fixed positions, respectively. In
the figure, there is one probe for each row of a two
dimensional array having a number of columns equal to the

length of the unknown   sequence, in this example 21. The
data obtained by separately hybridizing the 60 probes to the
21-mer are listed under the column, "Perfect Matches." The
data represent the number of each probe type having perfect
complementarity with a four base subsequence of the 21- mer.
Under the 21-mer sequence itself the probes are positioned
along the sequence where perfect complementarity occurs. The
objective of a reconstruction algorithm is to determine the
positions of enough probes so that the target sequence can be
reconstructed.

The reconstruction problem can be approached in many
ways. The problem is related to the traveling salesman
problem in that it involves finding a permutation of objects
which is in some sense optimal.   There is an extensive
literature on such combinatorial problems which provides
guidance in formulating the best approach for a particular
embodiment, e.g. Lawler   et al, eds., The Traveling Salesman
Problem: A Guided Tour of Combinatorial Optimization (John
Wiley & Sons, New York, 1985); Kirkpatrick, J. Stat. Phys.,
Vol. 34, pgs. 975-986 (1984); Held and Karp, J. Soc. Indust.
Appl. Math., Vol. 10, pgs. 196-210 (1962); and Lin and
Kernighan, Oper. Res., Vol 21, pgs. 498-516 (1973).
A preferred approach to the reconstruction problem requires
that the target sequence include one or more known sequence
regions. In particular, a first known   sequence region is
located at one end of the target sequence and a second known
sequence region is located at the other end of the target
sequence. The presence of the two known sequence regions
permits the construction of a simplified and efficient
reconstruction algorithm. Roughly, the reconstruction problem
is a problem of finding an ordering of overlapping probe
sequences which corresponds to the target sequence. The known
sequence regions define the starting and ending probes
sequences in a reconstruction. The intervening unknown
sequence region can be reconstructed from the remaining probe
sequences by requiring that each successively selected probe
properly overlap the previously selected probe sequence.

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Figure 2 is a flow chart of such an algorithm. It consists of
two parts which are performed alternatively, drawing probes
from the same set (referred to herein as the data set)
determined by the hybridization data: (1) construction of
candidate sequences from properly overlapping fixed-initial-
position and fixed-final position probes starting from one of
the known sequence regions, and (2) construction of candidate
sequences from properly overlapping fixed-final-position and
fixed-initial-position probes   starting from the other known
sequence region. The term "properly overlapping" simply means
overlapping in the sense described at the beginning of this
section and illustrated in Figure 1. Thus, in this algorithm
only probes having either a fixed initial position (3') or a
fixed final position (5'), or both, are employed in the
reconstruction. These two classes of probes are referred to
herein as FIP probes and FFP probes.

For the algorithm, two sets of numbers (or logical
variables depending on the implementation) are defined by the
nucleotide sequences of the first and second known sequence
regions. These sets are referred to as the initial left
register and the initial right register 2, respectively. The
size of the registers   depends on the length of the probes
employed. Usually the registers have L-1 elements, or
entries, where L is the length of the probe. Starting with
the initial right register, the algorithm compares the entries
of the register with every FFP and FIP probe that forms a
perfect match with the target sequence, 4. The comparison is
between bases 2 through L of the selected probe and the
numbers (or entries) 1 through L-1 of the right register.
That is, base at position 2 is compared to the entry at
position 1 of the right register, base at position 3 is
compared to the entry at position 2 of the right register, and
so on. Initially, as stated above, the entries of the
registers are determined by the bases of the first and
second known sequence regions. If the comparison results in
proper overlap in each of the L-1 positions, then the current
contents of the register are loaded into a new right register

and then the entries of the new right register are shifted to
the right one position. That is, entry 1 of the new right
register is moved to position 2, entry 2 is moved to position
3, and so on. Entry L-1 is discarded, and the fixed base at
the initial position of the probe (or some representation of
it) is loaded into position 1. Next, for the new right
register and selected probe to be retained for further
comparisons, an FFP probe must be found that properly overlaps
the register and the initial fixed base of the selected FIP
probe (unless of course the FIP probe is also an FFP probe) 8.
When such selections are made (i.e. 4 and 8) the selected
probe(s) are removed from the data set. The new right
register is saved along with and associated set of properly
overlapping FIP probes whose selections led to the current
register, and an associated set of properly overlapping FFP
probes.

    After each new right register is formed, one or more left
registers are formed, 16 and 18, by extending preexisting left
registers in substantially the same way as the right
registers, excepts that FFP probes are selected first and
positions 1-L of the FFP probe are compared to entries 1-L of
the left register. The FIP and FFP probes are selected from
the probes remaining in the data set. That is, any probes
previously selected to "extend" the right or left registers
cannot be selected. This also holds for right registers
formed in successive iterations after the first. As a result
of these comparisons, pairs of right and left registers are
formed, and associated with each pair are four sets of
probes, 20: (i) the set of FIP probes selected to extend the
right register, (ii) the set of FFP probes selected to
properly overlap the right register and FIP probe, (iii) the
set of FFP probes selected to extend the left register, and
(iv) the set of FIP probes selected to properly overlap the
left register and FFP probe. At each step i (see Figure 2),
M_{i+1} such pairs and associated sets are formed.

    The comparisons between probe and register positions are
carried out as follows. The register entries are always the

bases A, C, G, or T (or some representation thereof). The
probe positions are always occupied by a base or the absence
of a base. Recall from above that probes can be represented
by the notation, for example, A0AA000A. The 0's represent in
this case represent either C, G, T, or a degeneracy-reducing
analog thereof. In other words, the 0's represent "not A's".
The comparisons entail the determination of the truth value of
a base (from the register) and a base or a negative of a base
(from the probe being compared). For example, if the
register entry is A and the probe entry is "not T", then the
logical operation of "A AND not T" is logically true. Thus,
proper overlap exists. On the other hand, is the probe entry
is "not A", then the logical operation of "A AND not A" is
logically false. Thus, the overlap is improper and the probe
is rejected.

In successive steps, each of the M_i pairs of registers
are compared to probes of their respective data sets,
generating in turn, a set of M_{i+1} pairs of registers. With
each step the data set is reduced in size by two or more
probes, and the respective canditate sequences are increased
in size by one base each. When a register is compared to each
of the remaining probes in its associated data set and no
probe is found that properly overlaps, the register and its
associated sets are discarded **6-14**. The algorithm halts when
every probe in the data sets have been used (i.e., sorted into
one of the four associated sets). If more than one
candidate sequences are generated, or if it is desired to
check the consistency of the data, the same process of
repeated rounds of comparisons can be carried out starting
with the initial left register and set of probes that have
fixed final positions <u>and</u> a perfect match with the target
sequence **10-16**. In this case, entries 1 through L-1 of the
initial left register are compared with probe positions 1
through L-1, respectively, and successive registers, R_j after
the jth round of comparisons, are generated by shifting
current entries to the left and entering the final fixed base
of the properly overlapping probe to position L-1 of the new

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register. In a similar manner to that described above,
additional candidate sequences are generated, and are compared
to the ones previously generated. Only ones that occur in
both sets are retained 18. Further eliminations are possible
by requiring that all of the remaining non-fixed final and
non-fixed initial position probes find properly overlapping
positions within each of the candidates 20.

Preferably, the algorithm is implemented on a computer
with parallel processing capabilities. For example, the
algoithm of appendix I can be loaded onto each of the nodes of
a hypercube parallel processor, e.g. a 1024 node NCUBE/ten
computer (Ncube Corp., Beaverton, Oregon).

The above algorithm does not necessarily give a unique
solution in every case. Generally, regions of high frequency
repeats (e.g. ACACACAC..., GTCGTCGTC..., or the like) or
constant regions (e.g. AAAAAAA..., or the like) substantially
longer than the probe give rise to non-unique solutions. For
example, it is impossible to uniquely reconstruct (with the
above algoritm) target sequences which contain long stretches
of a single base type within which a few bases of a
different type are clustered. Thus, if 4-mer probes were used
to reconstruct a sequence with a stretch containing --
AAAAAAAAAAAAAAAAAAAAAGCATAAAAAAAAAAAAAAA--the position of GCAT
within the sequence of A's cannot be unequivocally
determined. In some cases, if alternative solutions are found
the correct sequence can be discerned by sequencing the non-
uniquely determined portions of the target sequence by
standard techniques.

Example I. Sequence Determination of the 119 Basepair
            Sca-Xmn Fragment of pUC19 with 8-mer Probes
A 119 basepair double stranded DNA is obtained by Xmn I
and Sca I restriction endonuclease digestion of the pUC19
plasmid, described by Yanisch-Perron et al, Gene, Vol. 33,
pgs. 103-119 (1985), and widely available commercially, e.g.
Bethesda Research Laboratories (Gaithersburg, MD). Large
scale isolation of pUC19 can be carried out by standard

procedures, e.g. as disclosed by Maniatis et al, <u>Molecular</u>
<u>Cloning: A Laboratory Manual</u>, (Cold Spring Harbor Laboratory,
New York, 1982) (i.e. alkali lysis followed by equilibrium
centrifugation in cesium chloride-ethidium bromide
gradient). Alternatively, purified pUC19 is purchased
commercially as needed, e.g. from Bethesda Research
Laboratories.

     1 mg pUC19 DNA is precipitated with 95% ethanol, dried,
and resuspended in 1 ml of <u>Sca</u>-<u>Xmn</u> restriction buffer (e.g.,
50 mM NaCl, 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10mM 2-
mercaptoethanol, 100 ug bovine serum albumin) for about 2.0
hours at 37°C. After stopping the reaction by adding 0.5 M
EDTA (pH 7.5), the restriction buffer is mixed with xylene
cyanol and loaded onto a 8 percent polyacrylamide gel for
electrophoresis. The band containing the 119 basepair
fragment is excised, and the DNA eluted as described by
Maniatis et al, page 178 (cited above).

     The fragments are resuspended at 1 ng/100 ul of 0.2 N
NaOH for 10 minutes, chilled, and mixed with an equal volume
of 10xSSC (1.5 M sodium chloride and 0.15 M sodium citrate).
100 ul samples of this fragment solution are pipetted into
the wells of slot-blotting apparatus, e.g. eleven 72-well
Minifold II micro-sample filtration manifolds, available from
Schleicher and Scheull, Keene, NH), each apparatus holding a
GeneScreen membrane that had been previously wetted for 15-
20 minutes in 1xSSC. After 2 hours, the solution is gently
sucked through the membranes, washed with 2xSSC, and allowed
to dry. After drying, the membranes are baked at 80°C for 2-4
hours. Before application of probe, the membranes are treated
with prehybridization mixture (10x Denhardt's with 0.5% SDS
for 1 hour at 60°C, followed by washing with 2xSSC).

     Probes for hybridization are synthesized by
phosphoramidite chemistry on an Applied Biosystems, Inc. model
380A DNA synthesizer. 4 X 196=784 mixed oligonucleotide
probes are employed, a probe for each kind of 8-mer sequence
having either a fixed initial position or a fixed final
position (see Appendix I). Non-fixed positions of the cytosine

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and adenosine subsets of probes are filled by deoxyadenosine and deoxyinosine and deoxycytosine and deoxyinosine, respectively. The probes are ^{32}P labelled following the T4 polynucleotide kinase protocol of Maxum and Gilbert (Meth Enzymol., Vol. 65, pgs. 497-560 (1980)), applied to the manifold wells at 18°C for 16 hours at a concentration of 1 ng/ml in 500 ul of hybridization mixture consisting of 5x Denhardt's, 5xSSPE, and 0.5% SDS. After hybridization the membranes are washed 3 times with 6xSSC containing 0.5% SDS at 12°C, followed by 2 rinses with 3.0 M Me$_4$NCl, 50 mM Tris-HCl (pH 8.0), 0.5% SDS at 12°C, and a final 2.0 minute wash in 3.0 M Me$_4$NCl, 50 mM Tris-HCl (pH 8.0), 0.5% SDS at 26-27°. After washing, the dried membranes are autoradiographed on XAR-5 film (or its equivalent) for 2-4 days. "Slots" on the developed film are analyzed on a LKB UltroScan XL Laser Densitometer, or like instrument.

Numbers of perfectly matched probes are determined by comparing the relative signal strengths of probes having the same degree of degeneracy. Also, because a double stranded target sequence is used, the values for probe number used in the reconstruction algorithm are the average of the signal for each probe type and its complement (with respect to the fixed bases). The sequence is reconstructed from the probe number data by program RCON8, whose source code is listed in the Appendix. RCON8 assumes that the eight base sequences on each end of the target sequence are known sequence regions. The program returns the noncoding sequence listed in a 3'-5' orientation from left to right.

Example II.   Sequence Determination of the 323 Basepair
               Pvu II Fragment of pUC19 Using 9-mer Probes

A 323 basepair double stranded DNA is one of two fragments obtained by Pvu II digestion of pUC19. The same procedure is followed as described in Example I for preparing the 323 basepair Pvu II fragments, denaturing them, and anchoring them to GeneScreen substrates. Pre-hybridization, hybridization, and wash protocols are the same, except that

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the final high temperature wash is carried out at 28-29°C.

Probes are synthesized and labeled as in Example I. 1556 probes are employed. A probe is prepared for each 9-mer sequence having a fixed base at the initial or final position (i.e. 389 each for A, C, G, and T probes). As in Example I, probes of the form 100000000 and 000000001 are replaced by three probes having different types of fixed bases, e.g. 00000000T is replaced by A0000000T, C0000000T, and G0000000T.

The sequence of the Pvu fragment is reconstructed with a modified version of the program of Appendix I which specifically accommodates 9-mer probe data. Like the 8-mer version, the program assumes that the nine base sequences on each end of the target sequence are known sequence regions.

Brief Description of the Drawings

Figure 1 illustrates the general problem of sequence reconstruction by showing how a 21-mer sequence can be reconstructed by four subsets of 4-mer probes.

Figure 2 is a flow chart diagrammatically illustrating a preferred reconstruction algorithm.

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Appendix I. <u>Source</u> <u>Code</u> <u>Listing</u> <u>of</u> <u>Reconstruction</u>
             <u>Algorithm</u> <u>RCON8</u>

```
       program RCON8
c
c          Program RCON8  reconstructs sequences from 8-mer
c             probes having fixed bases at their initial
c             positions and fixed bases at their final
c             positions.
c
       implicit integer*2 (a-z)
       dimension rl(xx,120),r2(xx,120),l1(xx,120),l2(xx,120)
       dimension dr2(xx,120),dll(xx,120),dl2(xx,120),drlnum(xx)
       dimension dr2num(xx),dllnum(xx),dl2num(xx),pset(240,2)
       dimension pnum8(4,259,8),rregl(xx,7),rreg2(xx,7)
       dimension lreg2(xx,7),lregl(xx,7)
       dimension tab(4,-4:4),drl(xx,120)
       dimension na(259),nc(259),ng(259),nt(259)
       character*1 seql(120),seqr(120),s,pseq8(4,259,8)      .
c
       character*10 pdata
       common rl,r2,ll,l2,drl,dr2,dll,dl2,rregl,rreg2,lregl,
     1         pnum8,tab,drlnum,dr2num,dllnum,dl2num,npx,np0,
     2         lreg2,pset,pseq8
c
c    READ pseq8 (list of all possible probe types) from
c             data file.
c    GENERATE pnum8 from pseq8.
c    READ probe data from data file and load into arrays
c             na, nc, ng, and nt.
c    GENERATE pset from pseq and na, nc, ng, and nt.
c    READ register transition table values from data file
c             and load into array tab.
c    ENTER bases of known sequence regions into arrays
c             rreg and lreg.
c
       numreg=1
c
c          NUMREG is the current number of rregisters
c          NPX is the number of probes in the data having
c             an initial (3') fixed base.
c          NPXX is the number of probes in the data having
c             both an initial (3') and final (5') fixed base.
c
       halt=int((npxx + (npx-npxx)/2)/2)
       rl(1,1)=0
       ll(1,1)=0
       drlnum(1)=0
```

```
           dllnum(l)=0
            ii=0
1000        ii=ii+1
c
c         ii indexes the round of comparisons.  ii is also
c         equal to the current length of candidate sequences.
c
           w=0
           do 1100 f=1,numreg
              do 1200 kk=1,npx-np0
                 if(ii.eq.1) then
                  if(kk.gt.1 .and. pset(kk,1).eq.pset(r2(w,ii),1)
     1                .and. pset(kk,2).eq.pset(r2(w,ii),2) .and.
     2                skipa.eq.1) goto 1200
                    skipa=0
                    do 1550 j=1,7
1550                 if(tab(rregl(f,j),pnum8(pset(kk,2),pset(kk,1),
     1                     j+1)).eq.0) goto 1200
                 else
c
                 do 1300 mm=1,ii-1
1300              if(kk.eq.rl(f,mm) .or. kk.eq.ll(f,mm)) goto 1200
                    do 1400 mm=1,dllnum(f)
1400                  if(kk.eq.dll(f,mm)) goto 1200
                    if(kk.gt.1 .and. pset(kk,1).eq.pset(r2(w,ii),1)
     1                  .and. pset(kk,2).eq.pset(r2(w,ii),2) .and.
     2                  skipa.eq.1) goto 1200
                    skipa=0
                 do 1500 j=1,7
1500                  if(tab(rregl(f,j),pnum8(pset(kk,2),pset(kk,1),
     1                     j+1)).eq.0) goto 1200
                 endif
c
                skipb=0
                 if(pnum8(pset(kk,2),pset(kk,1),8).lt.0 .or.
     1              pset(kk,1).le.3) then
                    do 1600 jj=npx-np0+1,npx
                     if(ii.eq.1) then
                 if(jj.gt.1 .and. pset(jj,1).eq.pset(dr2(w,dr2num(w)),
     1              1) .and. pset(jj,2).eq.pset(dr2(w,dr2num(w)),2)
     2                .and. skipb.eq.1) goto 1600
                       skipb=0
                        do 1950 x=1,8
                        if(x.eq.1) then
                    if(tab(pset(kk,2),pnum8(pset(jj,2),pset(jj,1),
     1                   1)).eq.0) goto 1600
                        else
                          if(tab(rregl(f,x-1),pnum8(pset(jj,2),
```

```
   1                                  pset(jj,1),x)).eq.0) goto 1600
                            endif
1950                        continue
                      else
                        do 1700 mm=1,drlnum(f)
1700                        if(jj.eq.drl(f,mm)) goto 1600
                        do 1800 mm=1,ii-1
1800                        if(jj.eq.ll(f,mm)) goto 1600
               if(jj.gt.1 .and. pset(jj,1).eq.pset(dr2(w,dr2num(w)),
   1               1) .and. pset(jj,2).eq.pset(dr2(w,dr2num(w)),2)
   2                  .and. skipb.eq.1) goto 1600
                        skipb=0
                        do 1900 x=1,8
                          if(x.eq.1) then
                     if(tab(pset(kk,2),pnum8(pset(jj,2),pset(jj,1),
   1                        1)).eq.0) goto 1600
                          else
                            if(tab(rregl(f,x-1),pnum8(pset(jj,2),
   1                          pset(jj,1),x)).eq.0) goto 1600
                          endif
1900                      continue
                      endif
                        ksave=kk
                        jsave=jj
                        w0=w
c
                        call left(ii,w,f,jsave,ksave)
c
                        if(w.eq.w0) goto 1600
                        do 2000 k=w0+1,w
                          do 2100 i=1,6
2100                        rreg2(k,i+1)=rregl(f,i)
                            rreg2(k,1)=pset(kk,2)
                          do 2600 q=1,drlnum(f)
2600                        dr2(k,q)=drl(f,q)
                          dr2num(k)=drlnum(f) + 1
                          dr2(k,dr2num(k))=jj
2000                      continue
                        if(ii.eq.1) then
                          do 2200 k=w0+1,w
2200                        r2(k,1)=kk
                        else
                          do 2300 k=w0+1,w
                            do 2400 x=1,ii-1
2400                          r2(k,x)=rl(f,x)
2300                          r2(k,ii)=kk
                        endif
                        skipa=1
```

```
                    skipb=1
        1600        continue
c
              else
c
                  jsave=0
                  ksave=kk
                  w0=w
c
                  call left(ii,w,f,jsave,ksave)
c
                  if(w.eq.w0) goto 1200
                  do 2700 k=w0+1,w
                      do 2800i=1,6
        2800            rreg2(k,i+1)=rreg1(f,i)
                      rreg2(k,1)=pset(kk,2)
                      do 2900 q=1,dr1num(f)
        2900            dr2(k,q)=dr1(f,q)
                      dr2num(k)=dr2num(f)
        2700        continue
                  if(ii.eq.1) then
                      do 6000 k=w0+1,w
        6000            r2(k,1)=kk
                  else
                      do 6100 k=w0+1,w
                          do 6200 x=1,ii-1
        6200                r2(k,x)=r1(f,x)
        6100            r2(k,ii)=kk
                  endif
                  skipa=1
              endif
        1200    continue
        1100  continue
c
                  numreg=w
                      do 7000 k=1,numreg
                          do 7100 m=1,ii
                              r1(k,m)=r2(k,m)
        7100                11(k,m)=12(k,m)
                          do 7200 m=1,7
                              rreg1(k,m)=rreg2(k,m)
        7200                lreg1(k,m)=lreg2(k,m)
                          do 7300 m=1,dr2num(k)
        7300                dr1(k,m)=dr2(k,m)
                          do 7400 m=1,dl2num(k)
        7400                dl1(k,m)=dl2(k,m)
                          dr1num(k)=dr2num(k)
                          dl1num(k)=dl2num(k)
```

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```
7000                     continue
              if (ii.lt.halt) goto 1000
c
c                     PRINT SEQUENCES
c
3000          continue
5000     end
c
c
         subroutine left(ii,w,f,jsave,ksave)
         implicit integer*2 (a-z)
         dimension r1(xx,120),r2(xx,120),l1(xx,120),l2(xx,120)
         dimension dr2(xx,120),dl1(xx,120),dl2(xx,120),dr1num(xx)
         dimension dr2num(xx),dl1num(xx),dl2num(xx),pset(240,2)
         dimension pnum8(4,259,8),rreg1(xx,7),rreg2(xx,7)
         dimension lreg2(xx,7),lreg1(xx,7)
         dimension tab(4,-4:4),dr1(xx,120)
         character*1 pseq8(4,259,8)
         common r1,r2,l1,l2,dr1,dr2,dl1,dl2,rreg1,rreg2,lreg1,
     1          pnum8,tab,dr1num,dr2num,dl1num,dl2num,npx,np0,
     2          lreg2,pset,pseq8
c
         skipa=0
         do 1000 hh=1,npx
         if(pnum8(pset(hh,2),pset(hh,1),8).lt.0 .or.
     1      pset(hh,1).le.3 .or. hh.eq.jsave) goto 1000
         if(ii.eq.1) then
            if(hh.gt.1 .and. pset(hh,1).eq.pset(l2(w,ii),1)
     1          .and. pset(hh,2).eq.pset(l2(w,ii),2) .and.
     2          skipa.eq.1) goto 1000
          skipa=0
            do 1350 j=1,7
1350        if(tab(lreg1(f,j),pnum8(pset(hh,2),pset(hh,1),j))
     1             .eq.0) goto 1000
         else
c
         do 1100 mm=1,ii-1
1100        if(hh.eq.r1(f,mm) .or. hh.eq.l1(f,mm)) goto 1000
            do 1200 mm=1,dr1num(f)
1200           if(hh.eq.dr1(f,mm)) goto 1000
            if(hh.gt.1 .and. pset(hh,1).eq.pset(l2(w,ii),1)
     1          .and. pset(hh,2).eq.pset(l2(w,ii),2) .and.
     2          skipa.eq.1) goto 1000
          skipa=0
          do 1300 j=1,7
1300        if(tab(lreg1(f,j),pnum8(pset(hh,2),pset(hh,1),j))
     1             .eq.0) goto 1000
         endif
```

```
c
                        skipb=0
                    if(pset(hh,1).ge.137) then
                        do 1400 rr=1,npx-np0
                        if(rr.eq.ksave) goto 1400
                        if(ii.eq.1) then
                    if(rr.gt.1 .and. pset(rr,1).eq.pset(dl2(w,dl2num(w)),
      1             1) .and. pset(rr,2).eq.pset(dl2(w,dl2num(w)),2)
      2                   .and. skipb.eq.1) goto 1400
                        skipb=0
                        do 1750 x=1,8
                            if(x.eq.8) then
                                if(tab(pnum8(pset(hh,2),pset(hh,1),x),
      1                          pnum8(pset(rr,2),pset(rr,1),x)).eq.0)
      2                            goto 1400
                            else
                            if(tab(lregl(f,x),pnum8(pset(rr,2),pset(rr,1),
      1                            x)).eq.0) goto 1400
                            endif
 1750                   continue
                        else
c
                        do 1500 mm=1,dllnum(f)
 1500                      if(rr.eq.dll(f,mm)) goto 1400
                        do 1600 mm=1,ii-1
 1600                      if(rr.eq.rl(f,mm)) goto 1400
                    if(rr.gt.1 .and. pset(rr,1).eq.pset(dl2(w,dl2num(w)),
      1             1) .and. pset(rr,2).eq.pset(dl2(w,dl2num(w)),2)
      2                   .and. skipb.eq.1) goto 1400
                        skipb=0
                        do 1700 x=1,8
                            if(x.eq.8) then
                                if(tab(pnum8(pset(hh,2),pset(hh,1),x),
      1                          pnum8(pset(rr,2),pset(rr,1),x)).eq.0)
      2                            goto 1400
                            else
                            if(tab(lregl(f,x),pnum8(pset(rr,2),pset(rr,1),
      1                            x)).eq.0) goto 1400
                            endif
 1700                   continue
                        endif
                        skipa=1
                        skipb=1
                        w=w+1
                        dl2num(w)=dllnum(f)+1
                        do 1710 k=1,dllnum(f)
 1710                     dl2(w,k)=dll(f,k)
                        dl2(w,dl2num(w))=rr
```

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```
c
              do 1800 i=1,6
1800              lreg2(w,i)=lreg1(f,i+1)
                  lreg2(w,7)=pset(hh,2)
              if(ii.eq.1) then
                  l2(w,1)=hh
              else
                  do 1900 k=1,ii-1
1900                  l2(w,k)=l1(f,k)
                      l2(w,ii)=hh
              endif
1400       continue
         else
           skipa=1
           w=w+1
           dl2num(w)=dl1num(f)
           do 2200 j=1,dl1num(f)
2200          dl2(w,j)=dl1(f,j)
           do 2000 i=1,6
2000          lreg2(w,i)=lreg1(f,i+1)
              lreg2(w,7)=pset(hh,2)
           if(ii.eq.1) then
               l2(w,1)=hh
           else
               do 2100 k=1,ii-1
2100               l2(w,k)=l1(f,k)
                   l2(w,ii)=hh
           endif
         endif
1000    continue
        return
        end
```

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I CLAIM:

1.   A method for determining the nucleotide sequence of
a nucleic acid, the method comprising the steps of:
     providing a set of probes, each probe within the
set having a predetermined length and each probe within
the set having a predetermined sequence of fixed and
non-fixed positions, the fixed positions comprising one
or more predetermined kinds of nucleotides;
     hybridizing the probes of the set to the nucleic
acid;
     determining the number of copies of each probe in
the set that form perfectly matched duplexes with the
nucleic acid; and
     reconstructing the nucleotide sequence of the
nucleic acid from the predetermined sequences of the
probes that form perfectly matched duplexes with the
nucleic acid.

2.   The method of claim 1 wherein said set contains at
least one probe comprising a sequence of fixed and non-
fixed positions equivalent to that of each permutation
of a plurality of fixed and non-fixed positions equal
to or less than the length of the probe.

3.   The method of claim 2 further including the steps
of:
     anchoring a known quantity of said nucleic acid to
each of a plurality of solid phase supports; and
washing each of the solid phase supports after
hybridizing said probes so that substantially all of
said probes not forming perfectly matched duplexes with
said nucleic acid are removed from the solid phase
support, and so that substantially all of said probes
forming perfectly matched duplexes with said nucleic
acid remain on the solid phase support.

4. The method of claim 3 wherein said step of hybridizing includes separately hybridizing each probe of said set to said nucleic acid on a different solid phase support of said plurality of solid phase supports.

5. The method of claim 4 wherein said predetermined length of said probes are in the range of from seven to eleven nucleotides, inclusive.

6. The method of claim 5 wherein said non-fixed positions of said probes are occupied by at least one degeneracy-reducing analog.

7. A method for determining the nucleotide sequence of a nucleic acid, the method comprising the steps of:
    providing a first set of probes, each probe within the first set having the same length, the length being from seven to ten nucleotides, and each probe within the first set having a predetermined sequence of fixed and non-fixed bases, the fixed bases being deoxyadenosine and the non-fixed bases comprising deoxycytosine, deoxygauanosine, thymidine, or a degeneracy-reducing analog thereof, such that for each permutation of fixed and non-fixed bases less than or equal to the length of the probe, the first set contains at least one probe having a sequence equivalent to such permutation;
    providing a second set of probes, each probe within the second set having the same length, the length being from seven to ten nucleotides, and each probe within the second set having a predetermined sequence of fixed and non-fixed bases, the fixed bases being deoxycytosine and the non-fixed bases comprising deoxyadenosine, deoxygauanosine, thymidine, or a degeneracy-reducing analog thereof, such that for each permutation of fixed and non-fixed bases less than or

equal to the length of the probe, the second set
contains at least one probe having a sequence
equivalent to such permutation;

providing a third set of probes, each probe
within the third set having the same length, the length
being from seven to ten nucleotides, and each probe
within the third set having a predetermined sequence of
fixed and non-fixed bases, the fixed bases being
deoxyguanosine and the non-fixed bases comprising
deoxyadenosine, deoxycytosine, thymidine, or a
degeneracy-reducing analog thereof, such that for each
permutation of fixed and non-fixed bases less than or
equal to the length of the probe, the third set
contains at least one probe having a sequence
equivalent to such permutation;

providing a fourth set of probes, each probe
within the fourth set having the same length, the
length being from seven to ten nucleotides, and each
probe within the fourth set having a predetermined
sequence of fixed and non-fixed bases, the fixed bases
being thymidine and the non-fixed bases comprising
deoxyadenosine, deoxycytosine, deoxyguanosine, or a
degeneracy-reducing analog thereof, such that for each
permutation of fixed and non-fixed bases the length of
the probe, the fourth set contains at least one probe
having a sequence equivalent to such permutation;

anchoring a known quantity of the nucleic acid to
each of a plurality of solid phase supports;

separately hybridizing each probe of the first,
second, third, and fourth sets to the nucleic acid
anchored on the solid phase supports;

washing each of the solid phase supports after
hybridizing said probes so that substantially all of
said probes not forming perfectly matched duplexes with
the nucleic acid are removed from the solid phase
support, and so that substantially all of said probes
forming perfectly matched duplexes with the nucleic

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acid remain on the solid phase support;

determining the number of copies of each probe in each set that form perfectly matched duplexes with the nucleic acid; and

reconstructing the nucleotide sequence of the nucleic acid from the predetermined sequences of the probes that form perfectly matched duplexes with the nucleic acid.

8.   The method of claim 7 wherein said nucleic acid contains at least one known sequence region.

9.   The method of claim 8 wherein said probe of said first set having said length from eight to nine nucleotides, said probe of said second set having said length from eight to nine nucleotides, said probe of said third set having said length from eight to nine nucleotides, and said probe of said fourth set having said length from eight to nine nucleotides.

10.   The method of claim 9 wherein said degeneracy-reducing analog of said first set includes deoxyinosine, 5-fluorodeoxyuridine, and N^4-methoxycytosine, said degeneracy-reducing analog of said second set includes deoxyinosine and 2-aminopurine, and said degeneracy-reducing analog of said third set includes 2-aminopurine and N^4-methoxycytosine.

11.   The method of claim 10 wherein said step of washing includes exposing said solid phase support to tetramethylammonium chloride at a concentration of between about 2 to 4 moles per liter.

TARGET SEQUENCE   PERFECT
PROBE   MATCHES

5'-C G A A T G G A A C T A C C G T A A C C T-3'

```
3'A000          A 0 0 0       A 0 0 0       A 0 0 0          3
  0A00        0 A 0 0       0 A 0 0     0 A 0 0              3
  00A0      0 0 A 0       0 0 A 0     0 0 A 0                3
  000A    0 0 0 A       0 0 0 A     0 0 0 A     0 0 0 A      4
  AA00                                                      0
  A0A0                                                      0
  A00A                                                      0
  0AA0                                                      0
  0A0A                                                      0
  00AA                                                      0
  AAA0                                                      0
  AA0A                                                      0
  A0AA                                                      0
  0AAA                                                      0
  AAAA                                                      0

  C000    C 0 0 0     C 0 0 0                 C 0 0 0        3
  0C00  0 C 0 0                           0 C 0 0            2
  00C0                                  0 0 C 0              1
  000C                                0 0 0 C                1
  CC00          C C 0 0                                     1
  COC0                                                      0
  C00C                                                      0
  0CC0        0 C C 0                                       1
  0C0C                                                      0
  00CC        0 0 C C                                       1
  CCC0                                                      0
  CC0C                                                      0
  C0CC                                                      0
  0CCC                                                      0
  CCCC                                                      0

  G000  G 0 0 0                       G 0 0 0                2
  0G00                      0 G 0 0                          1
  00G0                    0 0 G 0                            1
  000G                  0 0 0 G               0 0 0 G        2
  GG00                            G G 0 0                    1
  G0G0                                                      0
  G00G                    G 0 0 G                            1
  0GG0                      0 G G 0         0 G G 0          2
  0G0G                                                      0
  00GG                        0 0 G G       0 0 G G          2
  GGG0                                                      0
  GG0G                                                      0
  G0GG                                                      0
  0GGG                                                      0
  GGGG                                                      0

  T000        T 0 0 0         T 0 0 0       T 0 0 0          3
  0T00                      0 T 0 0                          1
  00T0                    0 0 T 0                            1
  000T        0 0 0 T               0 0 0 T                  2
  TT00      T T 0 0       T T 0 0             T T 0 0        3
  TOT0                                                      0
  TOOT                    T 0 0 T                            1
  0TT0    0 T T 0       0 T T 0             0 T T 0          3
  0T0T                                                      0
  00TT  0 0 T T       0 0 T T             0 0 T T            3
  TTT0                                                      0
  TTOT                                                      0
  TOTT                                                      0
  0TTT                                                      0
  TTTT                                                      0
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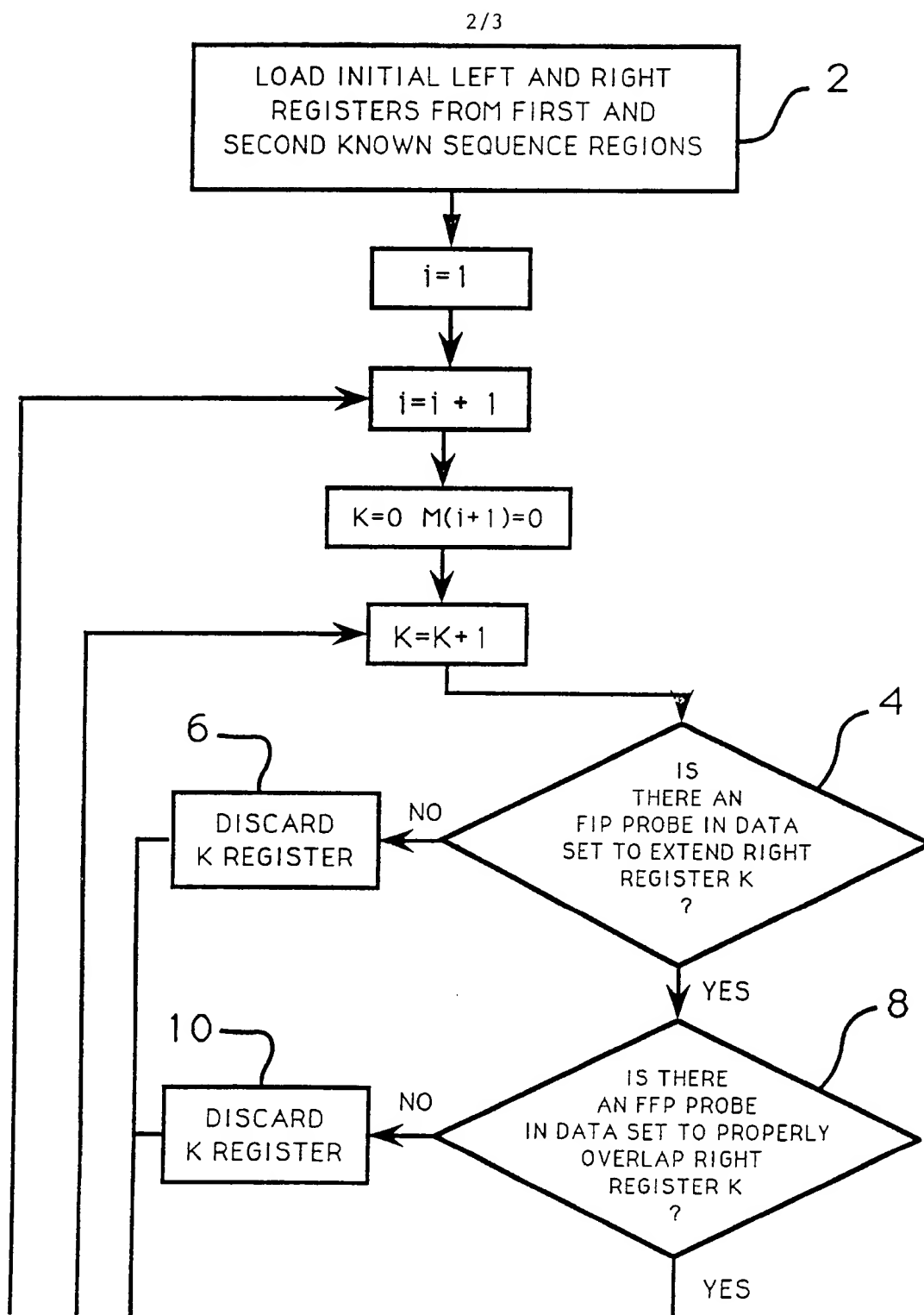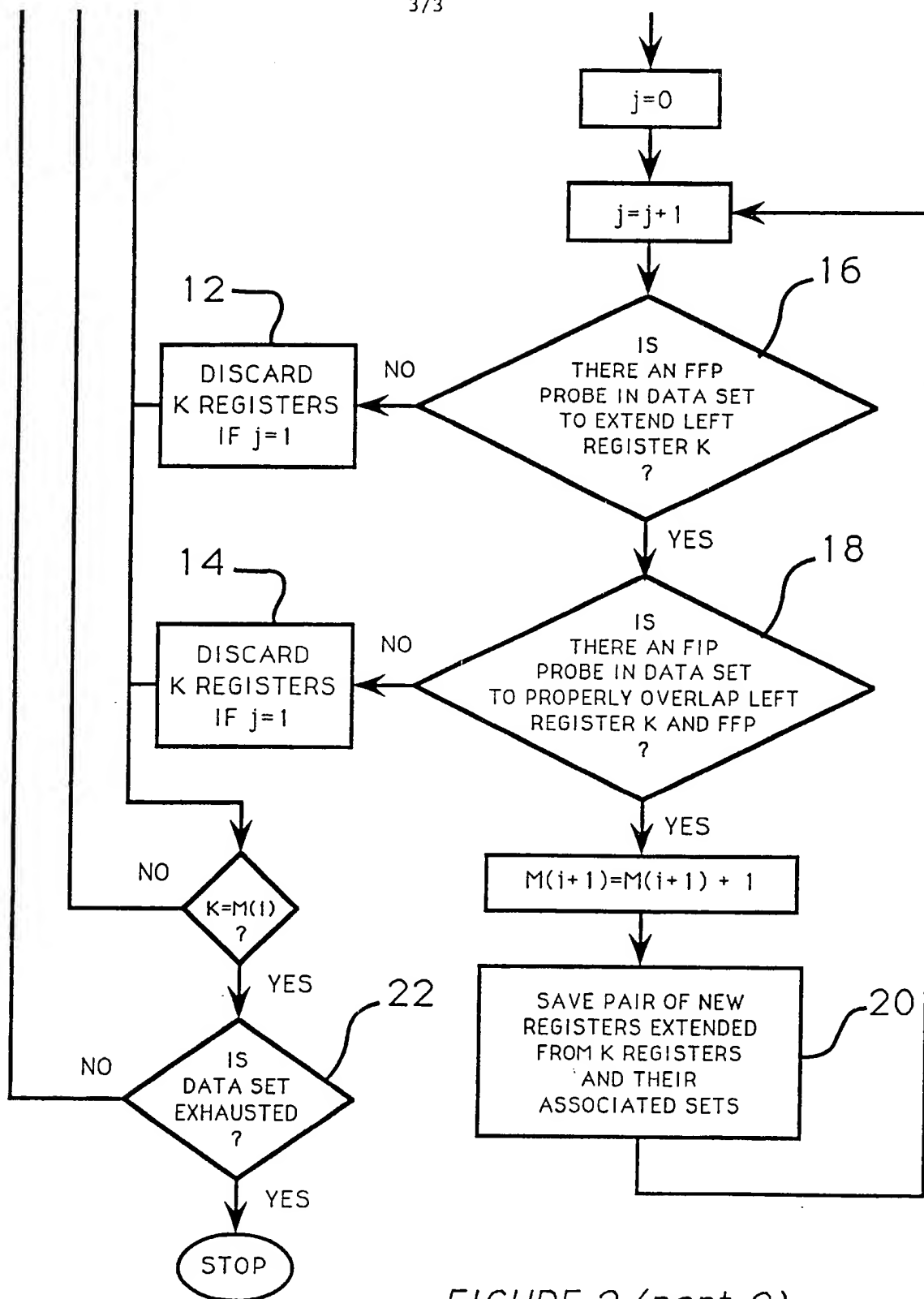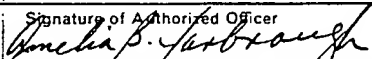
FIGURE 1

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```
┌─────────────────────────────────────┐
│   LOAD INITIAL LEFT AND RIGHT        │           2
│   REGISTERS FROM FIRST AND           │
│   SECOND KNOWN SEQUENCE REGIONS      │
└─────────────────────────────────────┘
                  │
                  ▼
            ┌──────────┐
            │  i = 1   │
            └──────────┘
                  │
                  ▼
            ┌──────────┐
            │ i = i + 1│◄────────
            └──────────┘
                  │
                  ▼
         ┌──────────────────┐
         │ K=0  M(i+1)=0    │
         └──────────────────┘
                  │
                  ▼
            ┌──────────┐
            │ K = K + 1│◄────────
            └──────────┘
                  │
                  ▼
```

6

┌──────────────┐   ╱╲
│   DISCARD   │ NO   IS   4
│ K REGISTER │◄──── THERE AN
└──────────────┘   FIP PROBE IN DATA
                   SET TO EXTEND RIGHT
                     REGISTER K
                         ?
                        ╲╱
                         │ YES
                         ▼
10   8

┌──────────────┐   ╱╲
│   DISCARD   │ NO   IS THERE
│ K REGISTER │◄──── AN FFP PROBE
└──────────────┘   IN DATA SET TO PROPERLY
                   OVERLAP RIGHT
                     REGISTER K
                         ?
                        ╲╱
                         │
                        YES

FIGURE 2 (part 1)

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FIGURE 2 (part 2)

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

```
IPC(5):   C12Q 1/68
U.S.CL.:   435/6
```

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. CL.	435/6, 436/501, 935/77,78

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

DNAX RESEARCH INSTITUTE OF MOLECULAR AND CELLULAR BIOLOGY, INC.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13.
Y	US, A, 4,613,566 POTTER, 23 September 1986, (See claims)	1-11
Y,P	US, A, 4,868,104, KURN et al., 19 September 1989, (See abstract).	1-11
Y	US, A, 4,710,465, WEISSMAN et al., 1 December 1987, (See claims)	1-11
Y	US, A, 4,689,295, TABER et al., 25 August 1987, (See claims and examples).	1-11
Y,P	US, A, 4,849,332, LORINCZ, 18 July 1989, (See claims).	1-11

* Special categories of cited documents: 10

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
0 6 FEB 1990	0 5 MAR 1990
International Searching Authority ISA/US	Signature of Authorized Officer AMELIA BURGESS YARBROUGH

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